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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
31671-176438

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
Unassigned **10/069541**

INTERNATIONAL APPLICATION NO.
PCT/JP00/05545

INTERNATIONAL FILING DATE
August 18, 2000

PRIORITY DATE CLAIMED
August 27, 1999

TITLE OF INVENTION
HIGH-AFFINITY CHOLINE TRANSPORTER

APPLICANT(S) FOR DO/EO/US
Tatsuya HAGA and Takashi OKUDA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau. (attach form IB 308)
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter 2 and 35 U.S.C. 1.821 - 1.825
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information: Paper copy of Sequence Listing (29 pages) and Petition for Full-tone Photographs (\$130.00)
- 20a. ☐ For purposes of examination, please insert the annexes to the IPER, so that the application will comprise the following pages of the English translation:
Specification: Original pages Amended pages
Claims: Original claims Amended claims

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Rec'd PCT/PTO 27 FEB 2002

10/069541

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Handwritten initials

In re application of:

Tatsuya HAGA et al.

Appl. No. Unassigned

Filed: February 27, 2002

For: HIGH-AFFINITY CHOLINE
TRANSPORTER

Int'l. Appln. No.: PCT/JP00/05545

Int'l. Filing Date: August 18, 2000

Atty. Docket No. 31671-176438

Customer No.



26694

PATENT TRADEMARK OFFICE

PETITION UNDER 37 CFR 1.84

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a Petition under Rule 84 (a)(2) to accept color photographs and illustrations in the above identified application for Figures 9 and 10. On information and belief, the claimed invention and explanation thereof is technically difficult to illustrate by black and white line drawings. On information and belief, color photographs and illustrations are necessary because they are the only practicable medium for illustrating the claimed invention and explanation thereof in a manner that will facilitate an understanding of the claimed invention.

In compliance with the Rule for submission of color photographs and illustrations, the formal drawings concurrently submitted include three (3) sets of color photographs. Additionally, the application includes the statement required by Rule 1.84 (a)(2) as the first paragraph in the Brief Description of the Drawing.

A check covering the \$130.00 Petition fee required by Rule 17(h) is attached. If no fee is attached or if the check is for an insufficient amount, authorization is given to charge our Deposit Account No. 22-0261 with any balance due and to credit our account with any overpayment.

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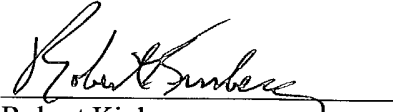
Applicant(s): Tatsuya HAGA et al.

In view of the above, it is requested that color photographs be permitted in the present application.

Date:

2/27/02

Respectfully submitted,



Robert Kinberg

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107-069547

JG13 Rec'd PCT/PTO 27 FEB 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Tatsuya HAGA et al.

Appl. No. Unassigned

Filed: February 27, 2002

For: HIGH-AFFINITY CHOLINE
TRANSPORTER

Int'l. Appln. No.: PCT/JP00/05545

Int'l. Filing Date: August 18, 2000

Atty. Docket No. 31671-176438

Customer No.



26694

PATENT TRADEMARK OFFICE

Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to calculation of the fees, please amend claims 24-27, 29-33, 35-38, 40-48, 50-52 and 56-63 attached to the specification as follows:

24. (Amended) A fusion protein being constructed by expressing a cDNA encoding fusion proteins of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 16.

25. (Amended) A fusion protein being constructed by expressing a cDNA encoding fusion proteins of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 18.

26. (Amended) A fusion protein being constructed by expressing a cDNA encoding fusion proteins of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 20.

27. (Amended) A fusion protein being constructed by expressing a cDNA encoding fusion proteins of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 22.

29. (Amended) An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 16.

30. (Amended) An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 18.

31. (Amended) An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 20.

32. (Amended) An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 22.

33. (Amended) The antibody according to claim 28, wherein the antibody is a monoclonal antibody.

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35. (Amended) A host cell containing an expression system which can express a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 16.

36. (Amended) A host cell containing an expression system which can express a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 18.

37. (Amended) A host cell containing an expression system which can express a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 20.

38. (Amended) A host cell containing an expression system which can express a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 22.

40. (Amended) A non-human animal whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 16.

41. (Amended) A non-human animal whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or overexpresses on its

Applicant(s): Tatsuya HAGA et al.

47. (Amended) A cell having high-affinity choline transporter activity being obtainable by the preparing method of a cell having high-affinity choline transporter activity according to claim 45.

48. (Amended) A screening method of a promoter or a suppressor of high-affinity choline transporter activity characterized in measuring/evaluating high-affinity choline transporter activity of the protein having high-affinity choline transporter activity according to claim 14 in the presence of a subject material.

50. (Amended) A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell membrane or the cell is measured/evaluated, wherein the cell membrane or the cell which expresses a protein having high-affinity choline transporter activity is the host cell containing an expression system which can express a protein having high-affinity choline transporter activity according to claim 34.

51. (Amended) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 48, wherein the protein having high-affinity choline transporter activity is a recombinant protein.

52. (Amended) A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell obtained from the non-human animal according to claim 39 is cultivated in vitro in the presence of a subject material; the activity and/or the expression

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amount of a protein having high-affinity choline transporter activity in the cell is measured/evaluated.

56. (Amended) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 52, wherein the non-human animal is a mouse or a rat.

57. (Amended) A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 48.

58. (Amended) A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 48.

59. (Amended) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the protein according to claim 14.

60. (Amended) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the protein according to claim 14.

61. (Amended) A diagnostic method for diseases relating to the expression or the activity of a high-affinity choline transporter characterized in comparing a DNA sequence encoding a high-affinity choline transporter in a sample to a DNA sequence encoding the protein according to claim 19.

62. (Amended) A diagnostic probe for Alzheimer's disease comprising a whole or a part of an antisense strand of DNA or RNA encoding the protein according to claim 19.

63. (Amended) A diagnostic drug for Alzheimer's disease characterized in containing the diagnostic probe according to claim 62.

Please add the following new claims:

64. (New) A preparing method of a cell having high-affinity choline transporter activity characterized in introducing the DNA according to claim 9 into a cell whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient on its chromosome.

65. (New) A preparing method of a cell having high-affinity choline transporter activity characterized in introducing the DNA according to claim 9 into a cell whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient on its chromosome, wherein the cell having high-affinity choline transporter activity is integrated with the DNA in its chromosome, and stably shows high-affinity choline transporter activity.

66. (New) A cell having high-affinity choline transporter activity being obtainable by the preparing method of a cell having high-affinity choline transporter activity according to claim 46.

67. (New) A cell having high-affinity choline transporter activity being obtainable by the preparing method of a cell having high-affinity choline transporter activity according to claim 65.

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68. (New) A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell membrane or the cell is measured/evaluated, wherein the cell membrane or the cell which expresses a protein having high-affinity choline transporter activity is the cell having high-affinity choline transporter activity according to claim 47.

69. (New) A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell membrane or the cell is measured/evaluated, wherein the cell membrane or the cell which expresses a protein having high-affinity choline transporter activity is the cell having high-affinity choline transporter activity according to claim 64.

70. (New) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 49, wherein the protein having high-affinity choline transporter activity is a recombinant protein.

71. (New) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 53, wherein the non-human animal is a mouse or a rat.

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72. (New) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 54, wherein the non-human animal is a mouse or a rat.

73. (New) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 55, wherein the non-human animal is a mouse or a rat.

74. (New) A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 49.

75. (New) A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 52.

76. (New) A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 53.

77. (New) A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 54.

78. (New) A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 55.

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79. (New) A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 49.

80. (New) A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 52.

81. (New) A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 53.

82. (New) A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 54.

83. (New) A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to claim 55.

84. (New) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 57 as an active component.

85. (New) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the material which promotes activity or

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expression of a protein having high-affinity choline transporter activity according to claim 74 as an active component.

86. (New) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 75 as an active component.

87. (New) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 76 as an active component.

88. (New) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 77 as an active component.

89. (New) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 78 as an active component.

90. (New) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline

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transporter, and containing the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 58 as an active component.

91. (New) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 79 as an active component.

92. (New) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 80 as an active component.

93. (New) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 81 as an active component.

94. (New) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 82 as an active component.

95. (New) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline

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transporter, and containing the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 83 as an active component.

96. (New) A diagnostic method for diseases relating to the expression or the activity of a high-affinity choline transporter characterized in comparing a DNA sequence encoding a high-affinity choline transporter in a sample to a DNA sequence encoding the protein according to claim 20.

97. (New) A diagnostic probe for Alzheimer's disease comprising a whole or a part of an antisense strand of DNA or RNA encoding the protein according to claim 20.

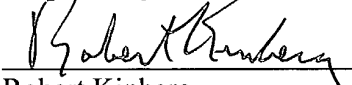
98. (New) A diagnostic drug for Alzheimer's disease characterized in containing the antibody according to claim 28.

REMARKS

This Preliminary Amendment is made to eliminate multiple claim dependency. Examination on the merits of the application is requested. A marked up version showing the changes made to the claims is attached.

Date: 2/27/02

Respectfully submitted,



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-13-

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MARKED-UP VERSION OF THE CLAIMS

24. (Amended) [The fusion protein according to claim 23] A fusion protein being constructed by expressing a cDNA encoding fusion protein of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim [15 or] 16.

25. (Amended) [The fusion protein according to claim 23] A fusion protein being constructed by expressing a cDNA encoding fusion protein of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim [17 or] 18.

26. (Amended) [The fusion protein according to claim 23] A fusion protein being constructed by expressing a cDNA encoding fusion protein of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim [19 or] 20.

27. (Amended) [The fusion protein according to claim 23] A fusion protein being constructed by expressing a cDNA encoding fusion protein of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim [21 or] 22.

29. (Amended) [The antibody according to claim 28] An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the

protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim [15 or] 16.

30. (Amended) [The antibody according to claim 28] An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim [17 or] 18.

31. (Amended) [The antibody according to claim 28] An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim [19 or] 20.

32. (Amended) [The antibody according to claim 28] An antibody which specifically binds to a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim [21 or] 22.

33. (Amended) The antibody according to [any one of claims 28 to 32] claim 28, wherein the antibody is a monoclonal antibody.

35. (Amended) [The host cell according to claim 34] A host cell containing an expression system which can express a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim [15 or] 16.

36. (Amended) [The host cell according to claim 34] A host cell containing an expression system which can express a protein having high-affinity choline transporter

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activity, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim [17 or] 18.

37. (Amended) [The host cell according to claim 34] A host cell containing an expression system which can express a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim [19 or] 20.

38. (Amended) [The host cell according to claim 34] A host cell containing an expression system which can express a protein having high-affinity choline transporter activity, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim [21 or] 22.

40. (Amended) [The non-human animal according to claim 39] A non-human animal whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim [15 or] 16.

41. (Amended) [The non-human animal according to claim 39] A non-human animal whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim [17 or] 18.

42. (Amended) [The non-human animal according to claim 39] A non-human animal whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, wherein the protein

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having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim [19 or] 20.

43. (Amended) [The non-human animal according to claim 39] A non-human animal whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim [21 or] 22.

44. (Amended) The non-human animal according to [any one of claims 39 to 43] claim 39, wherein the non-human animal is a mouse or a rat.

45. (Amended) A preparing method of a cell having high-affinity choline transporter activity characterized in introducing the gene according to [any one of claims 8 to 10] claim 8 into a cell whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient on its chromosome.

46. (Amended) [The preparing method of a cell having high-affinity choline transporter activity according to claim 45] A preparing method of a cell having high-affinity choline transporter activity characterized in introducing the gene according to claim 8 into a cell whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient on its chromosome, wherein the cell having high-affinity choline transporter activity is integrated with the gene [or the DNA according to any one of claims 8 to 10] in its chromosome, and stably shows high-affinity choline transporter activity.

47. (Amended) A cell having high-affinity choline transporter activity being obtainable by the preparing method of a cell having high-affinity choline transporter activity according to claim 45 [or 46].

Applicant(s): Tatsuya HAGA et al.

48. (Amended) A screening method of a promoter or a suppressor of high-affinity choline transporter activity characterized in measuring/evaluating high-affinity choline transporter activity of the protein having high-affinity choline transporter activity [according to any one of claims 14 to 22] claim 14 in the presence of a subject material.

50. (Amended) [The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 49] A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell membrane or the cell is measured/evaluated, wherein the cell membrane or the cell which expresses a protein having high-affinity choline transporter activity is the host cell containing an expression system which can express a protein having high-affinity choline transporter activity according to [any one of claims 34 to 38] claim 34], or is the cell having high-affinity choline transporter activity according to claim 47].

51. (Amended) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to [any one of claims 48 to 50] claim 48, wherein the protein having high-affinity choline transporter activity is a recombinant protein.

52. (Amended) A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell obtained from the non-human animal according to [any one of claims 39 to 44] claim 39 is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell is measured/evaluated.

Applicant(s): Tatsuya HAGA et al.

56. (Amended) The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to [any one of claims 52 to 55] claim 52, wherein the non-human animal is a mouse or a rat.

57. (Amended) A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to [any one of claims 48 to 56] claim 48.

58. (Amended) A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to [any one of claims 48 to 56] claim 48.

59. (Amended) A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the protein according to [any one of claims 14 to 22,] claim 14 [and/or the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 57] as an active component.

60. (Amended) A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the protein according to [any one of claims 14 to 22,] claim 14 [and/or the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 58] as an active component.

63. (Amended) A diagnostic drug for Alzheimer's disease characterized in containing the diagnostic probe according to claim 62 [and/or the antibody according to any one of claims 28 to 33].

10/ptz

SPECIFICATION

TITLE OF THE INVENTION

HIGH-AFFINITY CHOLINE TRANSPORTER

Technical Field

This invention relates to a protein having high-affinity choline transporter activity, a gene encoding said protein and the use of the same.

Prior Art

The autonomic nervous system which spreads to organs throughout a body and regulates the most basic functions of living organism including energy metabolism, circulation, respiration and reproduction along with endocrine system, is classified into the sympathetic and parasympathetic nervous systems. All autonomic nerve fibers excluding postganglionic fibers of the sympathetic nerve, motor nerve fiber, and sudoriferous gland/blood vessel dilative fiber in the sympathetic nerve are cholinergic, and acetylcholine is vital for the function of the autonomic nerve and the motor nerve. It has been known that the cholinergic neuron, being observed also in the brain, is important for recognizing function of the brain and that it degenerates after the onset of Alzheimer's disease. In the cholinergic neuron, because of lack of biosynthetic ability for choline, choline, an acetylcholine decomposition product, is taken up into a cell by a high-affinity choline transporter at the presynaptic terminals to be reused for synthesizing acetylcholine. The high-affinity choline uptake is a rate-limiting step for acetylcholine

synthesis and is presumed to regulate the efficiency of synaptic transmission (J. Neurochem. 18, 781-798, 1971, Science 178, 626-628, 1972, Biochem. Biophys. Acta 291, 564-575, 1973, Mol. Pharmacol. 9, 630-639, 1973, J. Pharmacol. Exp. Ther. 192, 86-94, 1975, J. Neurochem. 30, 15-21, 1978, J. Neurochem. 44, 11-24, 1985, J. Neurochem. 60, 1191-1201, 1993, J. Neurochem. 20, 581-593, 1973, Eur. J. Pharmacol. 102, 369-370, 1984). To date, most of cDNAs of transporters for major neurotransmitters have been isolated, however, a cDNA of the high-affinity choline transporter, which is physiologically important, has not been identified.

Disclosure of the Invention

So far, the existence of a protein being localized in the cholinergic neuron and having a function of taking up choline, a precursor of acetylcholine, into a cell has been expected, but molecular properties of said protein, a high-affinity choline transporter, have been unknown. An object of the present invention is to provide a physiologically important protein having the high-affinity choline transporter activity, a gene which encodes the protein, and a screening method of a high-affinity choline transporter activity promoter using the protein, the gene and the like.

The inventors have conducted intensive study to attain the above-mentioned object: with information of genomic project (Science 282, 2012-2018, 1998), Na⁺-dependent transporter cDNAs being expected from the genomic sequence of a nematode (C. elegans) were cloned one by one, and the high-affinity choline uptake activity of each cDNA was examined in the oocyte expression system of Xenopus, and the cDNA of nematode

high-affinity choline transporter (cho-1) was identified on the basis of the above examination, then homologous molecules (CHT1) were cloned from rat spinal cord by using the homology of a base sequence to the cDNA as an index. This CHT1 had no homology to neurotransmitter transporters (J. Neurochem. 71, 1785-1803, 1998), but had 20 to 25% homology to molecules which belong to Na⁺-dependent glucose transporter family (Nature 330, 379-381, 1987).

Northern blot analysis revealed that transcripts of CHT1 were confirmed only in spinal cord, basal forebrain, corpus striatum and brain stem, and CHT1 seemed to be expressed in cholinergic neurons. Accordingly, CHT1 was expressed in oocytes of *Xenopus*. As a result, choline uptake activity that is Na⁺-dependent and completely inhibited by hemicholinium-3 was observed. These results indicate that CHT1 has high-affinity choline transporter activity. Further, the inventors have cloned choline transporter cDNAs derived from a human and from a mouse, and determined their base sequences, and have confirmed that their expression products have high-affinity choline uptake activity. The present invention has thus completed.

The present invention relates to a gene which encodes a protein having high-affinity choline transporter activity (claim 1), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 2, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having high-affinity choline transporter activity (claim 2), DNA containing a base sequence represented

by Seq. ID No. 1 or its complementary sequence and a part or a whole of these sequences (claim 3), DNA derived from a nematode which hybridizes with DNA comprising a gene according to claim 3 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 4), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 4, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having high-affinity choline transporter activity (claim 5), DNA containing a base sequence represented by Seq. ID No. 3 or its complementary sequence and a part or a whole of these sequences (claim 6), DNA derived from a rat which hybridizes with DNA comprising a gene according to claim 6 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 7), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 6, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having high-affinity choline transporter activity (claim 8), DNA containing a base sequence represented by Seq. ID No. 5 or its complementary sequence and a part or a whole of these sequences (claim 9), DNA derived from a human which hybridizes with DNA comprising a gene according to claim 9 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 10), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq.

ID No. 8, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.8, and having high-affinity choline transporter activity (claim 11), DNA containing a base sequence represented by Seq. ID No. 7 or its complementary sequence and a part or a whole of these sequences (claim 12), and DNA derived from a mouse which hybridizes with DNA comprising a gene according to claim 12 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 13).

The present invention also relates to a protein having high-affinity choline transporter activity (claim 14), a protein comprising an amino acid sequence represented by Seq. ID No. 2 (claim 15), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having nematode high-affinity choline transporter activity (claim 16), a protein comprising an amino acid sequence represented by Seq. ID No. 4 (claim 17), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having rat high-affinity choline transporter activity (claim 18), a protein comprising an amino acid sequence represented by Seq. ID No. 6 (claim 19), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having human high-affinity choline transporter activity (claim 20), a protein comprising an amino acid sequence represented by Seq. ID No. 8 (claim 21), and a protein comprising an amino acid

sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.8, and having mouse high-affinity choline transporter activity (claim 22).

The present invention further relates to a fusion protein being constructed by expressing a cDNA encoding fusion proteins of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag (claim 23), the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 24), the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18 (claim 25), the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 26), and the fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 27).

The present invention still further relates to an antibody which specifically binds to a protein having high-affinity choline transporter activity (claim 28), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 29), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has rat

high-affinity choline transporter activity according to claim 17 or 18 (claim 30), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 31), the antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 32), and the antibody according to any one of claims 28 to 32, wherein the antibody is a monoclonal antibody (claim 33).

The present invention also relates to a host cell containing an expression system which can express a protein having high-affinity choline transporter activity (claim 34), the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 35), the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18 (claim 36), the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 37), and the host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 38).

The present invention further relates to a non-human animal in which function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or

overexpresses on its chromosome (claim 39), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16 (claim 40), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18 (claim 41), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20 (claim 42), the non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22 (claim 43), and the non-human animal according to any one of claims 39 to 43, wherein the non-human animal is a mouse or a rat (claim 44).

The present invention still further relates to a preparing method of a cell having high-affinity choline transporter activity characterized in introducing the gene or the DNA according to any one of claims 8 to 10 into a cell whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient on its chromosome (claim 45), the preparing method of a cell having high-affinity choline transporter activity according to claim 45, wherein the cell having high-affinity choline transporter activity is integrated with the gene or the DNA according to any one of claims 8 to 10 in its chromosome, and stably shows high-affinity choline transporter activity (claim 46), and a cell having high-affinity choline transporter activity being obtainable by

the preparing method of a cell having high-affinity choline transporter activity according to claim 45 or 46 (claim 47).

The present invention also relates to a screening method of a promoter or a suppressor of high-affinity choline transporter activity characterized in measuring/evaluating high-affinity choline transporter activity of the protein having high-affinity choline transporter activity according to any one of claims 14 to 22 in the presence of a subject material (claim 48), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell membrane or the cell is measured/evaluated (claim 49), the screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 49, wherein the cell membrane or the cell which expresses a protein having high-affinity choline transporter activity is the host cell containing an expression system which can express a protein having high-affinity choline transporter activity according to any one of claims 34 to 38, or is the cell having high-affinity choline transporter activity according to claim 47 (claim 50), the screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to any one of claims 48 to 50, wherein the protein having high-affinity choline

transporter activity is a recombinant protein (claim 51), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell obtained from the non-human animal according to any one of claims 39 to 44 is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell is measured/evaluated (claim 52), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity (claim 53), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity (claim 54), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, and then evaluating the activity and/or the expression amount of

a protein having high-affinity choline transporter activity in comparison with the case using wild-type non-human animal (claim 55), and the screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to any one of claims 52 to 55, wherein the non-human animal is a mouse or a rat (claim 56).

The present invention further relates to a material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to any one of claims 48 to 56 (claim 57), a material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to any one of claims 48 to 56 (claim 58), a medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the protein according to any one of claims 14 to 22, and/or the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 57 as an active component (claim 59), and a medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the protein according to any one of claims 14 to 22, and/or the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 58 as an active component (claim 60).

The present invention still further relates to a diagnostic method for diseases relating to the expression or the activity of a high-affinity choline transporter characterized in comparing a DNA sequence encoding a high-affinity choline transporter in a sample to a DNA sequence encoding the protein according to claim 19 or 20 (claim 61), a diagnostic probe for Alzheimer's disease comprising a whole or a part of an antisense strand of DNA or RNA encoding the protein according to claim 19 or 20 (claim 62), and a diagnostic drug for Alzheimer's disease characterized in containing the diagnostic probe according to claim 62 and/or the antibody according to any one of claims 28 to 33 (claim 63).

Brief Explanation of the Drawings

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Fig. 1 is a view showing the result of [³H] choline uptake of oocytes from *Xenopus* of the present invention being injected with nematode cho-1 (C48D1.3 cRNA) or water.

Fig.2 is a view showing the result of the effect of Na⁺ on choline uptake of oocytes from *Xenopus* of the present invention being injected with nematode cho-1 (C48D1.3 cRNA) or water.

Fig.3 is a view showing the result of the HC3-induced inhibition of choline uptake of oocytes from *Xenopus* of the present invention being injected with nematode cho-1 (C48D1.3

to the membrane prepared from COS7 cells being introduced with CHT1 cDNA of the present invention or vector pcDNA 3.1 separately.

Fig.16 is a view showing the result of saturation analysis of specific [^3H] HC3 binding to the membrane prepared from COS7 cells being introduced with CHT1 cDNA of the present invention or vector pcDNA 3.1 separately.

Fig.17 is a view showing the result of displacement of specific [^3H] HC3 binding by HC3 of the present invention, choline (Cho), acetylcholine (ACh).

Best Mode for Carrying out the Invention

The cDNA of nematode high-affinity choline transporter of the present invention, being described in Seq. ID No. 1, can be obtained by injecting each cRNA prepared from candidate full-length cDNAs, which are expected as a member of Na^+ -dependent transporter family according to C. elegans genome project, into oocytes of Xenopus, and examining the uptake of choline. The high-affinity uptake of choline in brain synaptosomes of mammals was completely inhibited by 1 μM hemicholinium-3 (HC3) ($K_i=10\text{-}100\text{ nM}$), while the low-affinity uptake of choline, which is distributed in every cells, was inhibited only by HC3 with higher concentration ($K_i=50\text{ }\mu\text{M}$). Therefore, the sensitivity to 1 μM HC3 can be used as criteria of high-affinity choline uptake during the process. For example, it is possible to confirm the identification, the expression, and the localization of an object gene from the candidate cDNA of a nematode (C. elegans) as follows.

It has been found that cDNA corresponding to the gene expected as C48D1.3 promotes significant choline uptake, being

of L1 larva possessing cho-1::gfp reporter DNA at the outside of chromosome is shown as Fig. 5 (scale bar; 50 μ m). In Fig. 5, the arrowhead indicates nerve ring. In the ventral nerve cord, GFP is expressed only in cholinergic motor nerve, however, some of DA, DB nerve cells do not express GFP owing probably to deficiency of reporter DNA at the outside of chromosome. It supports the idea that cho-1 is a high-affinity choline transporter of the cholinergic neuron.

The cDNA of rat high-affinity choline transporter of the present invention, being described in Seq. ID No. 3, can be prepared, for example, by a method comprising the steps of: paying attention to cho-1 homologous molecules of vertebrates and searching data base with amino acid sequences expected from cho-1, and identifying one candidate (GenBank accession number: AQ 316435) in human genomic survey sequence (GSS); amplifying cDNA fragments from rat spinal cord cDNA by PCR with degenerate primers on the basis of homology of base sequences between the human genome DNA and cho-1; screening rat spinal cord cDNA library with this fragment, and a positive cDNA clone was obtained. A protein with 580 amino acid residues showing 51% identity and 70% similarity to cho-1 was expected from the base sequence of the longest reading frame (see Fig. 4). This rat cDNA clone was designated as CHT1. In Fig. 4, each amino acid sequence of rat CHT1 and nematode CHO-1 is shown, and the identical and the similar residues are indicated on a black ground and a gray ground respectively. The expected transmembrane region I-XII is underlined. This protein represented by Seq. ID No. 4 can be constructed by a usual method.

The above-mentioned amino acid sequence of CHT1 is significantly homologous to members of Na⁺-dependent glucose

transporter family (20 to 25%). The phylogenetic tree of Na⁺-dependent glucose transporter family made by neighbor-joining method using a program CLUSTALW of National Institute of Genetics (Mishima, Japan) is shown in Fig. 6. In Fig. 6, the percentage of the identical amino acids, being contained in each protein, to rat CHT1 is shown on the right side. On the other hand, no homology was observed to a yeast choline transporter (J. Biol. Chem. 265, 15996-16003, 1990), a creatine transporter which had been originally reported as a high-affinity choline transporter (Biochem. Biophys. Res. Commun. 198, 637-645, 1994), and other neurotransmitter transporters.

The expected topology of CHT1 is thought to be the same as that of nematode CHO-1 fundamentally. Fig. 7 shows the expected topology of rat CHT1. In Fig. 7, the closed circles indicate the identical residues, the shadowed circles indicate highly conserved residues, and open circles indicate nonsimilar residues. The offshoots indicate the expected glycosylation sites. P among the circles shows the expected parts of phosphorylation induced by protein kinase C.

Next, the distribution of CHT1 mRNA expression was examined by Northern blot analysis and *in situ* hybridization. The expression of transcripts with the length of about 5 kb was confirmed by Northern blot analysis of various tissues of rats. Fig. 8 shows the result of Northern blot analysis of mRNA transcript of CHT1 in rat tissue, and the length of RNA standard (0.24 to 9.5 kb; GIBCO BRL) is exhibited on the left side. As shown in Fig. 8, an abundance of transcripts were confirmed in basal forebrain, brain stem and spinal cord, and a little of those were confirmed in corpus striatum. These tissues are known to contain cholinergic neurons. On the other hand, no

transcript was observed in other regions of the brain or in tissues of non-nervous systems.

Consistent with these results, *in situ* hybridization confirmed the expression of CHT1 mRNA in cell groups of main cholinergic neurons including corpus striatum, cell population in basal forebrain and ventral horn in spinal cord. Fig. 9 and 10 (scale bar; 1 mm) show micrographs of sections in bright-field, which were hybridized with a cRNA probe of an antisense labeled by digoxigenin. These micrographs relate to *in situ* hybridization analysis of CHT1 transcripts in rat brain and spinal cord. Fig. 9 indicates that mRNA transcripts of CHT1 were detected in vertical and horizontal limbs of the diagonal band (VDB, HDB), medial septal nucleus (MS), caudate and putamen (Cpu), and olfactory tubercle (Tu). Fig. 10 indicates that the expression was observed in ventral horn (VH) in spinal cord. Further, the adjacent section hybridized with a probe of vesicle acetylcholine transporter showed essentially same distribution. This expression distribution is essentially same as the reported distribution of cholineacetyl group transferase or vesicle acetylcholine transporter. These results show that the expression of CHT1 mRNA is limited to cholinergic neurons.

Next, choline uptake of CHT1 was examined by using oocytes of *Xenopus*. The choline uptake of the oocytes injected with CHT1 cRNA was 2 times to 4 times more than that of controls injected with water. Fig. 11 shows the result of [³H] choline uptake of oocytes of *Xenopus* injected with CHT1 cRNA or water. In Fig. 11, the open and the closed columns respectively indicate choline uptake in the standard solutions containing 100 mM NaCl or LiCl, and each column is shown by mean \pm SEM (n=6

to 8 oocytes). The effect of choline concentration on choline uptake is shown in Fig. 12. In Fig. 12, choline uptake of oocytes injected with water was subtracted from that of oocytes injected with cRNA in order to figure out CHT1-induced choline uptake, and the choline uptake was fitted to Michaelis-Menten curve. As shown in Fig. 12, choline uptake of CHT1 saturated when increasing choline concentration ($K_m = 2.2 \pm 0.2 \mu\text{M}$, $n=3$). The K_m of endogenous choline uptake of control is higher than $10 \mu\text{M}$.

Then, the result of HC3-induced inhibition of choline uptake is shown in Fig. 13. Fig. 13 indicates that choline uptake of CHT1 is completely inhibited by $0.1 \mu\text{M}$ HC3 ($K_i = 2-3 \text{ nM}$), whereas $10 \mu\text{M}$ HC3 induced only slight inhibition in control. As shown in Fig. 14, ion-dependency of choline uptake of CHT1 was examined and found to be Cl^- -dependent as well as Na^+ -dependent. The closed and the open columns indicate choline uptake of oocytes injected with water and with cRNA respectively (100 mM NaCl in the standard solution is substituted with 100 mM of each salt) shown in the figure. These results indicate that CHT1 has the characteristics expected from high-affinity choline uptake in brain synaptosomes (high-affinity to choline, high sensitivity to HC3, and Na^+ - Cl^- -dependency) (J. Neurochem. 27, 93-99, 1976).

In addition, [^3H] HC3 binding activity of membranes prepared from COS7 cells introduced with CHT1 cDNA and a vector (control) respectively was examined. The result is shown in Fig. 15. As Fig. 15 indicates, Na^+ -dependent [^3H] HC3 binding was observed in a membrane of a cell where CHT1 was expressed, but not in a control membrane. Subsequently, a saturation analysis was conducted for specific [^3H] HC3 binding. As shown

in Fig. 16, equilibrium dissociation constant (K_d) was estimated to be $1.6 \pm 0.2 \mu\text{M}$ ($n=3$). This value was similar to that reported in brain synaptosomes (J. Neurochem. 60, 1191-1201, 1993, Life Sci. 35, 2335-2343, 1984, Brain Res. 348, 321-330, 1985). Further, displacement of specific [^3H] HC3 binding by HC3, choline (Cho) and acetylcholine (ACh) was examined. Acetylcholine was measured in the presence of $1 \mu\text{M}$ physostigmine. The result is shown in Fig. 17. Fig. 17 indicates that specific [^3H] HC3 binding was displaced when the concentration of choline was at least about 10 times lower than that of acetylcholine. These results show that CHT1 is a HC3 binding site as well as a high-affinity choline transporter.

The cDNA of human high-affinity choline transporter of the present invention, being represented by Seq. ID No.5, can be prepared, for example, as follows: data base search was conducted with the amino acid sequence of nematode (*C. elegans*) CHO-1 to find a sequence of specific human genome DNA fragment having significant homology (R-107P12, a clone of human genomic survey sequence; GenBank accession number: AQ316435); a gene-specific primers for PCR were designed based on a base sequence of said DNA fragment; 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE were conducted using Marathon-Ready™ cDNA (Clontech) of human whole brain, together with an attached adapter primer; the obtained PCR product was cloned into a cloning vector for PCR, and a base sequence of inserted DNA was determined. In addition, an amino acid sequence expected from this DNA sequence is represented by Seq. ID No. 6. A protein having human high-affinity choline transporter activity represented by said Seq. ID No. 6 can be constructed by a usual method on the basis of DNA sequence information shown in Seq.

ID No. 5.

The cDNA of mouse high-affinity choline transporter of the present invention, being represented by Seq. ID No.7, can be prepared, for example, as follows: data base search was conducted with the amino acid sequence of nematode (*C. elegans*) CHO-1 to find a sequence of specific human genome DNA fragment having significant homology (R-107P12, a clone of human genomic survey sequence; GenBank accession number: AQ316435); a gene-specific primer for PCR was designed based on a base sequence of said DNA fragment; 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE were conducted using Marathon-Ready™ cDNA (Clontech) of mouse whole brain, together with an attached adapter primer; the obtained PCR product was cloned into a cloning vector for PCR, and a base sequence of inserted DNA was determined. In addition, an amino acid sequence expected from this DNA sequence is represented by Seq. ID No. 8. A protein having mouse high-affinity choline transporter activity represented by said Seq. ID No. 8 can be constructed by a usual method on the basis of DNA sequence information shown in Seq. ID No. 7.

Examples of a protein having high-affinity choline transporter activity of the present invention include a protein derived from natural materials and a recombinant protein. In addition to the ones represented by Seq. ID Nos. 2, 4, 6 and 8, which are specifically disclosed above, a protein comprising an amino acid sequence wherein one or a few amino acids are deficient, substituted or added in amino acid sequences represented by Seq. ID Nos. 2, 4, 6 and 8, and having high-affinity choline transporter activity is also included. These proteins can be prepared by known methods. Further, examples

of a gene or DNA encoding a protein having high-affinity choline transporter activity of the present invention include, in addition to the ones represented by Seq. ID Nos. 1, 3, 5 and 7, which are specifically disclosed above, a gene or DNA which encodes a protein comprising an amino acid sequence wherein one or a few amino acids are deficient, substituted or added in amino acid sequences represented by Seq. ID Nos. 2, 4, 6 and 8, and having high-affinity choline transporter activity, and DNA which encodes a protein hybridizing with said gene or DNA under a stringent condition and having high-affinity choline transporter activity. These genes and DNAs can be prepared by known methods.

Cholinergic neurons play an extremely important role in learning and memory. The damage of these neurons correlates to severity of dementia. The rate-limiting step in acetylcholine synthesis is presumed to be the uptake of choline, and its activity is controlled by neural activity or various kinds of stimuli. In the brains of patients who suffer Alzheimer's disease, the hyperfunction of high-affinity choline uptake and of HC3 binding activity are observed (Trends Neurosci. 15, 117-122, 1992, Ann. NY Acad. Sci. 777, 197-204, 1996, J. Neurochem. 69, 2441-2451, 1997). Cloning of said gene or DNA encoding a protein having high-affinity choline transporter activity and said protein having high-affinity choline transporter activity is important for elucidating the molecular mechanism of the high-affinity choline transporter and for developing new therapies for Alzheimer's disease.

The fusion protein of the present invention means a substance constructed by binding a protein from a nematode, a rat, a human, a mouse, etc., which has high-affinity choline

transporter activity, to a marker protein and/or a peptide tag. As the marker protein, any conventionally known marker protein can be used and the specific examples are alkaline phosphatase, Fc region of an antibody, HRP, and GFP. Conventionally known peptide tags, such as Myc tag, His tag, FLAG tag, GST tag, are exemplified as specific examples of the peptide tag of the present invention. Said fusion proteins can be constructed by a usual method, and are useful for the purification of a protein having high-affinity choline transporter activity utilizing the affinity between Ni-NTA and His tag, the detection of a protein having high-affinity choline transporter activity, the quantitation of an antibody to a protein having high-affinity choline transporter activity, and as a diagnostic marker for Alzheimer's disease, and an investigational reagent in the field concerned.

As an antibody that specifically combines with a protein having high-affinity choline transporter activity of the present invention, an immunospecific antibody such as a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a single stranded antibody, a humanized antibody and the like are concretely exemplified. Though these antibodies can be constructed by a usual method with the above-mentioned protein having high-affinity choline transporter activity as an antigen, a monoclonal antibody is more preferable among them because of its specificity. Said antibody that specifically binds to a protein having high-affinity choline transporter activity, such as a monoclonal antibody or the like, is useful, for instance, for the diagnosis of Alzheimer's disease, and for elucidation of molecular mechanism of a high-affinity choline transporter.

An antibody to a protein having high-affinity choline transporter activity is developed by administering fragments containing the protein having high-affinity choline transporter activity or its epitope, or cells that express said protein on the surface of the membrane, to animals (preferably excluding human) with usual protocol. For instance, a monoclonal antibody can be prepared by an arbitrary method that brings antibodies developed by cultured materials of continuous cell line, such as hybridoma method (Nature 256, 495-497, 1975), trioma method, human B-cell hybridoma method (Immunology Today 4, 72, 1983), and EBV-hybridoma method (MONOCLONAL ANTIBODIES AND CANCER THERAPY, pp. 77-96, Alan R. Liss, Inc., 1985).

In order to develop a single stranded antibody to the above-mentioned protein having high-affinity choline transporter activity of the present invention, the preparation method of single stranded antibodies (US Patent No. 4,946,778) can be applied. Further, in order to express a humanized antibody, it is possible to use transgenic mice, other mammalian animals or the like, and to isolate and identify the clones that express a protein having high-affinity choline transporter activity with the above-mentioned antibodies, and to purify the polypeptide by affinity chromatography. An antibody to a protein having high-affinity choline transporter activity could be used, in particular, for the diagnosis and the medical treatment of Alzheimer's disease, and the like.

This invention relates to a host cell which contains an expression system that can express said protein having high-affinity choline transporter activity. The gene that encodes a protein having high-affinity choline transporter activity can be introduced into a host cell by a number of methods

described in many standard laboratory manuals such as by Davis et al. (BASIC METHODS IN MOLECULAR BIOLOGY, 1986), and by Sambrook et al. (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989). Examples of those methods include calcium phosphate transfection, DEAE-dextran-mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection. Examples of the host cells include bacterial procaryotic cells such as *Escherichia coli*, *Streptomyces*, *Bacillus subtilis*, *Streptococcus*, *Staphylococcus* and the like; fungous cells such as yeast, *Aspergillus* and the like; insect cells such as *Drosophila* S2, *Spodoptera* Sf9 and the like; and animal or plant cells such as L cells, CHO cells, COS cells, HeLa cells, C127 cells, BALB/c3T3 cells (including mutant strains deficient in dihydrofolate reductase, thymidine kinase or the like), BHK21 cells, HEK293 cells, Bowes melanoma cells and the like.

As the expression system, any expression system that can express a protein having high-affinity choline transporter activity in a host cell will suffice. Examples of the expression system include expression systems derived from chromosome, episome and virus, for example, vectors derived from bacterial plasmid, yeast plasmid, papovavirus like SV40, vaccinia virus, adenovirus, chicken pox virus, pseudorabies virus, or retrovirus, vectors derived from bacteriophage, transposon, and the combination of these, for instance, vectors derived from genetic factors of plasmid and of bacteriophage such as cosmid or phagemid. These expression systems may contain a regulatory sequence that acts not only as a promoter

but also as a controller of expressions.

A host cell that contains the above-mentioned expression system, cell membrane of said host cell, and a protein having high-affinity choline transporter activity which is obtainable by the cultivation of said host cell can be used in the screening method of the present invention as hereinafter described. For example, the method of F. Pietri-Rouxel et al. (Eur. J. Biochem., 247, 1174-1179, 1997) or the like can be used as the method to obtain cell membranes, and publicly known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic-interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and lectin chromatography, preferably high-speed liquid chromatography can be used to pick up said protein having high-affinity choline transporter activity from cell cultured material and purify it. As columns used for affinity chromatography, in particular, there are columns to which a protein antibody having anti-high-affinity choline transporter activity is bound, or in case that a normal peptide tag is added to said high-affinity choline transporter, there are columns to which materials having affinity to the peptide tag are bound. Proteins having high-affinity choline transporter activity can be obtained by using these columns.

In the present invention, said non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient on its chromosome means a non-human animal wherein a part or a whole of a gene encoding a protein having high-affinity choline transporter activity on chromosome is inactivated by gene mutation such as

disruption, deficiency, substitution, etc. and function of expressing a protein having high-affinity choline transporter activity is lost. In addition, a non-human animal that overexpresses function of a gene that encodes a protein having high-affinity choline transporter activity on its chromosome means a non-human animal that produces larger amount of a protein having high-affinity choline transporter activity than a wild-type non-human animal does. Though specific examples of a non-human animal of the present invention include rodents, such as mice, rats and the like, a non-human animal of the present invention is not limited to these animals.

Homozygous non-human animals generated according to Mendelian ratio include a deficient type or an overexpression type for a protein having high-affinity choline transporter activity, and their littermate wild-type, and it is possible to carry out precise comparative experiments in individual level by using the deficient types, the overexpression types and the littermate wild-types of these homozygous non-human animals at the same time. Therefore, it is preferable to use animals of the same species, more preferably the littermates, as the wild-type non-human animals, in other words, the non-human animals being deficient in or overexpressing the function of a gene that encodes a protein having high-affinity choline transporter activity on their chromosome together in, for example, the screening hereinafter described in the present invention. The generating method of the non-human animals being deficient or overexpressing the function of a gene that encodes a protein having high-affinity choline transporter activity on their chromosome will be explained below, with an example of knockout mice and transgenic mice of a protein having

high-affinity choline transporter activity.

For example, a mouse being deficient in the function of a gene that encodes a protein having high-affinity choline transporter activity on its chromosome, in other words, a knockout mouse of a protein having high-affinity choline transporter activity on its chromosome can be constructed as follows. A gene that encodes a protein having high-affinity choline transporter activity is screened by using a gene fragment obtained from mouse gene library by a method like PCR. The screened gene that encodes a protein having high-affinity choline transporter activity is subcloned with a viral vector or the like, and specified by DNA sequencing. A target vector is constructed by substituting a whole or a part of a gene of this clone that encodes a protein having high-affinity choline transporter activity with pMC1 neo gene cassette or the like, and by introducing a diphtheria toxin A fragment (DT-A) gene, a herpes simplex virus thymidine kinase (HSV-tk) gene or other such genes into 3'-terminal side.

This constructed target vector is linearized and introduced into ES cells by electroporation or the like to induce homologous recombination. The ES cells wherein homologous recombination is induced by an antibiotic such as G418, ganciclovir (GANC) or the like are selected from the homologous recombinants. It is preferable to confirm whether the selected ES cells are the recombinants of the object by Southern blot or the like. A chimeric mouse is constructed by microinjecting a clone of the confirmed ES cells into a blastocyst of a mouse and then transplanting the blastocyst into a recipient mouse. A heterozygous mouse can be obtained by intercrossing the chimeric mouse with a wild-type mouse, and

invention. As an example of a method for preparing these cells of the present invention, a method wherein a whole or a part of said gene or DNA of the present invention is introduced into a cell being deficient in the function of a gene that encodes a protein having high-affinity choline transporter activity on its chromosome by transfection or the like to obtain a cell having high-affinity choline transporter activity is exemplified. As the cell having high-affinity choline transporter activity, in particular, it is preferable to use a cell wherein said gene or DNA is integrated into a chromosome and high-affinity choline transporter activity is exhibited stably.

By using the above-mentioned gene or DNA that encodes a protein having high-affinity choline transporter activity, a protein having high-affinity choline transporter activity, a fusion protein created by combining a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag, an antibody to a protein having high-affinity choline transporter activity, a host cell which contains an expression system that can express a protein having high-affinity choline transporter activity, a cell having high-affinity choline transporter activity, or the like, it becomes possible to screen a pharmaceutical material useful for the treatment of symptoms as in Alzheimer's disease or the like, in other words, a material that promotes or suppresses the activity or the expression of a high-affinity choline transporter.

Examples of the screening method of the present invention are: a method wherein the high-affinity choline transporter activity of the above-mentioned protein having high-affinity

choline transporter activity of the present invention is measured/evaluated in the presence of a subject material; a method wherein a cell membrane or a cell which expresses a protein having high-affinity choline transporter activity of the present invention is cultivated in vitro in the presence of a subject material, and the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell is measured/evaluated; and a method wherein a subject material is administered to said non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome and/or a wild-type non-human animal and then the activity and/or the expression amount of a protein having high-affinity choline transporter activity of the present invention is measured/evaluated. As said cell membrane or said cell, a cell such as a primary cultured cell obtained from said non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome or a wild-type non-human animal etc., a host cell containing an expression system which can express a protein having high-affinity choline transporter activity of the present invention, a cell having high-affinity choline transporter activity of the present invention, and cell membranes of these cells can be specifically exemplified.

The screening methods with said subject material and said protein having high-affinity choline transporter activity are now specifically explained together with examples, but the screening methods of the present invention are not limited to these examples. Cells expressing a protein having high-affinity choline transporter activity are cultured in the

presence of a subject material, and the increase or the decrease of a protein having high-affinity choline transporter activity expressed on the cell surface after a certain period of cultivation can be immunochemically detected by ELISA or other such method with an antibody that specifically combines to a protein having high-affinity choline transporter activity of the present invention, or can be evaluated by using suppression or promotion of mRNA expression as an index. The mRNA can be detected by methods such as DNA chip, Northern hybridization or the like. Moreover, with a cell to which a gene wherein luciferase or other such reporter genes is linked to downstream of promoter of a gene that encodes high-affinity choline transporter is introduced, the suppression or the promotion of the expression of a gene that encodes a protein having high-affinity choline transporter activity induced by a subject material can be detected by using the activity of said reporter gene as an index.

The present invention further relates a medical constituent being used for medical treatment for a patient who needs promotion of the activity or the expression of a protein having high-affinity choline transporter, or a medical constituent being used for medical treatment for a patient who needs suppression of the activity or the expression of a protein having high-affinity choline transporter, wherein the material contains a protein having high-affinity choline transporter activity, a material which promotes the activity or the expression of a protein having high-affinity choline transporter activity, or a material which suppresses activity or expression of a protein having high-affinity choline transporter activity as an active component. As a protein

having high-affinity choline transporter activity is involved in many biological functions including many pathological ones, it is expected that a compound that can stimulate a protein having high-affinity choline transporter activity and a compound being able to inhibit the function of said protein can be used as pharmaceuticals.

As the material which promotes or suppresses the activity or the expression of a protein having high-affinity choline transporter activity, any material can be used as long as it binds to a protein having high-affinity choline transporter activity, or works on a signal transmitting molecule on upstream, and then promotes the activity or the expression of a protein having high-affinity choline transporter activity or inhibits/antagonizes the activity or the expression of the protein by itself. Specific examples include an antibody, a ligand of a protein having high-affinity choline transporter activity, a fragment of said protein, and an oligonucleotide encoding said fragment, and these materials can be used as pharmaceuticals for treatment, prevention or the like of symptoms observed in the case of Alzheimer's disease or other such diseases, but use of them is not limited to the above examples.

The present invention also relates to a diagnostic method for diseases relating to the activity or the expression of a protein having high-affinity choline transporter activity comprising a comparison of a DNA sequence encoding a protein having high-affinity choline transporter activity in a sample with a DNA sequence encoding a protein having high-affinity choline transporter activity of the present invention. The mutant type of DNA which encodes a protein having high-affinity

choline transporter activity can be detected by finding gene-mutated individuals in DNA level, and this is useful for diagnosis of diseases caused by underexpression, overexpression or mutant expression of a protein having high-affinity choline transporter activity. Specific examples of a sample of said detection include cells of trial subjects, for example, genomic DNA, RNA or cDNA obtained from biopsy of blood, urine, saliva, tissue or the like, however said sample is not limited to these examples. It is also possible to use said sample being amplified by PCR or other such methods. Deficiency and insertion mutation of base sequences can be detected by the size change of the amplified product observed in comparison with normal genotype, and point mutation can be identified by hybridizing amplified DNA with a labeled gene that encodes a protein having high-affinity choline transporter activity. Thus, diagnosis or judgement of symptoms observed in the case of Alzheimer's disease or other such diseases can be made by detecting the mutation of a gene that encodes a protein having high-affinity choline transporter activity.

The present invention further relates to a diagnostic probe for diseases showing symptoms similar to those of Alzheimer's disease or the like comprising a whole or a part of an antisense chain of DNA or RNA encoding a protein having high-affinity choline transporter activity, and a diagnostic drug for diseases showing symptoms similar to those of Alzheimer's disease containing the diagnostic probe and/or an antibody which specifically binds to a protein having high-affinity choline transporter activity of the present invention. Said diagnostic probe is not limited in particular, as long as it comprises a whole or a part of an antisense chain of DNA (cDNA)

or RNA (cRNA) encoding a protein having high-affinity choline transporter activity and being long enough to be a probe (at least 20 bases). In order to make a diagnostic drug for symptoms similar to those of Alzheimer's disease containing said probe and/or an antibody which specifically binds to a protein having high-affinity choline transporter activity of the present invention as active components, it is preferable to dissolve said probe into an appropriate buffer or sterilizing water for preventing said probe from decomposition. Further, it is also possible to diagnose diseases showing symptoms similar to those of Alzheimer's disease by methods using these diagnostic drugs, such as immunostaining (Dev. Biol. 170, 207-222, 1995, J. Neurobiol. 29, 1-17, 1996), in situ hybridization (J. Neurobiol. 29, 1-17, 1996), in situ PCR or the like.

Experimental methods or the like of the above-mentioned various experiments will now be explained in more detail below.

(Cloning of high-affinity choline transporter cDNA)

The candidate cDNA of nematode high-affinity choline transporter was isolated from poly (A)+RNA of nematode mixture from various stages in the development by reverse transcription PCR and 3' RACE. MarathonTM cDNA Amplification Kit (Clontech) was used according to its protocol. A primer for sense direction of PCR was designed at a provisional translation initiating point of a predicted gene based on a DNA base sequence obtained from *C. elegans* genomic project. The amplified PCR product was subcloned into Nco I (smoothing) site and Not I site of a modified pSPUTK vector (Stratagene), and the base sequence of inserted DNA was determined. CHT1 cDNA of rat was isolated from rat spinal cord cDNA library by using GeneTrapper cDNA

Positive Selection System (GIBCO Bio-Rad Laboratory: GIBCO BRL) according to its protocol. The primer used was designed from the base sequence of a cDNA fragment obtained by degenerated PCR. The obtained cDNA clones were analyzed. Among them, positive clones were selected and subcloned into pSPUTK vector and pcDNA3.1+ vector (Invitrogen Corporation).

(Expression in oocytes of Xenopus)

In the presence of cap analog, cRNA was synthesized in vitro with SP6 or T7 RNA polymerase. 20 to 30 ng capped RNA was microinjected into oocytes (stage V to VI) of Xenopus. The uptake was measured in basically same manner as described previously (Nature 360, 467-471, 1992). Two or three days after the injection of RNA, choline uptake was conducted for 30 to 60 min. with oocytes (6 to 8) in 0.75 ml standard solution (0.01 to 1 μ M [3 H]-choline, 100 mM NaCl, 2mM KCl, 1mM MgCl₂, 1mM CaCl₂, 10mM HEPES, 5mM Tris: pH 7.4). The oocytes completing uptake were solubilized with 10% SDS, and the amount of [3 H] was measured by a liquid scintillation counter.

(GFP expression construct)

The transcriptional fusion construct of cho-1::gfp was constructed by PCR in same manner as described previously (Gene 212, 127-135, 1998). A gene that encodes a green fluorescent protein (GFP) located on downstream of a nuclear localization signal sequence (NLS) was inserted into a position 3 residues downstream of cho-1 translation initiating point so that the reading frame was fitted. NLS and gfp gene were amplified from pPD104.53 vector. In order to prepare 5.1 kb upstream region of cho-1 translation initiating point, a PCR primer being

designed to encompass the first 3 amino acid residues of cho-1 was used. By the same method as previously described (EMBO J. 10, 3959-3970, 1991), rol-6 (sul006) marker and generated DNA were injected into gonads of a nematode simultaneously.

(Northern blot analysis)

6 µg poly(A)+RNA prepared from various tissues of rats was separated by formaldehyde-agarose electrophoresis, and transferred to a nylon membrane, then hybridized with CHT1 cDNA fragment being labeled with [³²P] by random prime method in hybridization solution (solution containing the final concentration of 50% formamido, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 100 µg/ml salmon sperm DNA) at 42° C for 16 hours. The nylon membrane was washed under final condition (0.1 × SSPE, 0.1% SDS: 65° C), and then autoradiography was conducted for 7 days together with an enhancing screen.

(In situ hybridization)

The transcript of an antisense labeled with digoxigenin was synthesized in vitro. Alkaline hydrolysis was repeated for the transcripts until their mean length was prepared to be 200 to 400 b. Cryostat sections of fresh frozen tissue (10 to 20 µm) were used. Hybridization was conducted with labeled cRNA probe (about 1 µg/ml) dissolved in 1 × Denhardt's solution [solution containing the final concentration of 50 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 0.3 M NaCl, 50% formamido, 10% dextran sulphate, 1 mg/ml E. coli tRNA] at 45° C for 20 hours. Then the sections were washed twice in 2 × SSC/50% formamido and once in 1 × SSC/50% formamido, at 45° C respectively. The hybridized probe was visualized by using anti-digoxigenin Fab fragment

(Boehringer-Mannheim) and NBT/BCIP substrate. The sections were brought into reaction in substrate solution for 24 to 48 hours.

(Binding assay)

[³H] hemicholinium-3 (HC3; 128Ci/mmol) was obtained from NEN Life Science Products. Either pcDNA3.1-CHT1 or pcDNA3.1 was transiently expressed in COS7 cells respectively. TransFast Reagent (Promega) was introduced and used according to the protocol. Membranes were prepared by following steps: homogenizing cells in 0.32 M sucrose; centrifuging the cells for 1 hour at 200,000g; and suspending the precipitate. Binding assay was conducted in basically same manner as described previously. Specific binding amount was calculated by subtracting non-specific binding amount determined in the presence of 10 μ M HC3 from the whole binding amount. The K_d value was figured out by analyzing specific [³H] HC3 binding amount from data of saturation binding assay with nonlinear approximation.

Industrial Applicability

The present invention makes it possible to provide a protein having high-affinity choline transporter activity, which is physiologically important, and gene DNA encoding said protein. In addition, by using the said protein and gene DNA, it becomes possible to screen materials being useful for prevention or treatment of Alzheimer's disease, and to prepare cells being useful for gene therapy.

What Is Claimed Is:

1. A gene which encodes a protein having high-affinity choline transporter activity.

2. A gene which encodes a protein (a) or (b) described below;
(a) a protein comprising an amino acid sequence represented by Seq. ID No. 2,

(b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having high-affinity choline transporter activity.

3. DNA containing a base sequence represented by Seq. ID No. 1 or its complementary sequence and a part or a whole of these sequences.

4. DNA derived from a nematode which hybridizes with DNA comprising a gene according to claim 3 under a stringent condition, and encodes a protein having high-affinity choline transporter activity.

5. A gene which encodes a protein (a) or (b) described below;
(a) a protein comprising an amino acid sequence represented by Seq. ID No. 4,

(b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having high-affinity choline transporter activity.

6. DNA containing a base sequence represented by Seq. ID No. 3 or its complementary sequence and a part or a whole of these sequences.

7. DNA derived from a rat which hybridizes with DNA comprising a gene according to claim 6 under a stringent condition, and encodes a protein having high-affinity choline transporter activity.

8. A gene which encodes a protein (a) or (b) described below;
 (a) a protein comprising an amino acid sequence represented by Seq. ID No. 6,
 (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having high-affinity choline transporter activity.

9. DNA containing a base sequence represented by Seq. ID No. 5 or its complementary sequence and a part or a whole of these sequences.

10. DNA derived from a human which hybridizes with DNA comprising a gene according to claim 9 under a stringent condition, and encodes a protein having high-affinity choline transporter activity.

11. A gene which encodes a protein (a) or (b) described below;
 (a) a protein comprising an amino acid sequence represented by Seq. ID No. 8,
 (b) a protein comprising an amino acid sequence where one or

a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.8, and having high-affinity choline transporter activity.

12. DNA containing a base sequence represented by Seq. ID No. 7 or its complementary sequence and a part or a whole of these sequences.

13. DNA derived from a mouse which hybridizes with DNA comprising a gene according to claim 12 under a stringent condition, and encodes a protein having high-affinity choline transporter activity.

14. A protein having high-affinity choline transporter activity.

15. A protein comprising an amino acid sequence represented by Seq. ID No. 2.

16. A protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having nematode high-affinity choline transporter activity.

17. A protein comprising an amino acid sequence represented by Seq. ID No. 4.

18. A protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having rat

high-affinity choline transporter activity.

19. A protein comprising an amino acid sequence represented by Seq. ID No. 6.

20. A protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having human high-affinity choline transporter activity.

21. A protein comprising an amino acid sequence represented by Seq. ID No. 8.

22. A protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.8, and having mouse high-affinity choline transporter activity.

23. A fusion protein being constructed by expressing a cDNA encoding fusion proteins of a protein having high-affinity choline transporter activity and a marker protein and/or a peptide tag.

24. The fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16.

25. The fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has

rat high-affinity choline transporter activity according to claim 17 or 18.

26. The fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20.

27. The fusion protein according to claim 23, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22.

28. An antibody which specifically binds to a protein having high-affinity choline transporter activity.

29. The antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16.

30. The antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18.

31. The antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20.

32. The antibody according to claim 28, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22.

33. The antibody according to any one of claims 28 to 32, wherein the antibody is a monoclonal antibody.

34. A host cell containing an expression system which can express a protein having high-affinity choline transporter activity.

35. The host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16.

36. The host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18.

37. The host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20.

38. The host cell according to claim 34, wherein the protein having high-affinity choline transporter activity has mouse

high-affinity choline transporter activity according to claim 21 or 22.

39. A non-human animal whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome.

40. The non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has nematode high-affinity choline transporter activity according to claim 15 or 16.

41. The non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has rat high-affinity choline transporter activity according to claim 17 or 18.

42. The non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has human high-affinity choline transporter activity according to claim 19 or 20.

43. The non-human animal according to claim 39, wherein the protein having high-affinity choline transporter activity has mouse high-affinity choline transporter activity according to claim 21 or 22.

44. The non-human animal according to any one of claims 39 to 43, wherein the non-human animal is a mouse or a rat.

45. A preparing method of a cell having high-affinity choline transporter activity characterized in introducing the gene or the DNA according to any one of claims 8 to 10 into a cell whose function of a gene which encodes a protein having high-affinity choline transporter activity is deficient on its chromosome.

46. The preparing method of a cell having high-affinity choline transporter activity according to claim 45, wherein the cell having high-affinity choline transporter activity is integrated with the gene or the DNA according to any one of claims 8 to 10 in its chromosome, and stably shows high-affinity choline transporter activity.

47. A cell having high-affinity choline transporter activity being obtainable by the preparing method of a cell having high-affinity choline transporter activity according to claim 45 or 46.

48. A screening method of a promoter or a suppressor of high-affinity choline transporter activity characterized in measuring/evaluating high-affinity choline transporter activity of the protein having high-affinity choline transporter activity according to any one of claims 14 to 22 in the presence of a subject material.

49. A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell membrane or a cell which expresses a protein having high-affinity choline transporter

activity is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell membrane or the cell is measured/evaluated.

50. The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to claim 49, wherein the cell membrane or the cell which expresses a protein having high-affinity choline transporter activity is the host cell containing an expression system which can express a protein having high-affinity choline transporter activity according to any one of claims 34 to 38, or is the cell having high-affinity choline transporter activity according to claim 47.

51. The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression according to any one of claims 48 to 50, wherein the protein having high-affinity choline transporter activity is a recombinant protein.

52. A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell obtained from the non-human animal according to any one of claims 39 to 44 is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell is measured/evaluated.

53. A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity.

54. A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity.

55. A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal whose function of a gene encoding a protein having high-affinity choline transporter activity is deficient or overexpresses on its chromosome, and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity in comparison with the case using wild-type non-human animal.

56. The screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-

affinity choline transporter expression according to any one of claims 52 to 55, wherein the non-human animal is a mouse or a rat.

57. A material which promotes activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to any one of claims 48 to 56.

58. A material which suppresses activity or expression of a protein having high-affinity choline transporter activity being obtainable by the screening method according to any one of claims 48 to 56.

59. A medical constituent characterized in being used for a medical treatment for a patient who needs elevation of the activity or enhancement of the expression of a high-affinity choline transporter, and containing the protein according to any one of claims 14 to 22, and/or the material which promotes activity or expression of a protein having high-affinity choline transporter activity according to claim 57 as an active component.

60. A medical constituent characterized in being used for medical treatment for a patient who needs suppression of the activity or the expression of a high-affinity choline transporter, and containing the protein according to any one of claims 14 to 22, and/or the material which suppresses the activity or the expression of a protein having high-affinity choline transporter activity according to claim 58 as an active

Abstract

The present invention provides a protein having high-affinity choline transporter activity which is important physiologically, a gene encoding the protein, and a method of screening a material promoting the high-affinity choline transporter activity with the use of the same, and the like. By examining high-affinity choline uptake activity of Na⁺-dependent transporter cDNA deduced from the genomic sequence of a nematode (*C.elegans*) in a *Xenopus* oocyte expression system, the cDNA (*cho-1*) of nematode high-affinity choline transporter is identified. Then the cDNA (*CHT1*) of rat high-affinity choline transporter is cloned from rat spinal cord by using the homology of a base sequence to this cDNA as an index. Similarly, the cDNA of human high-affinity choline transporter is cloned from human genome.

Fig. 1

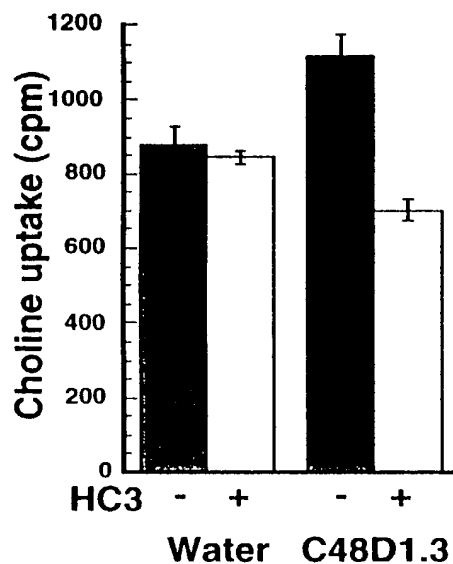


Fig. 2

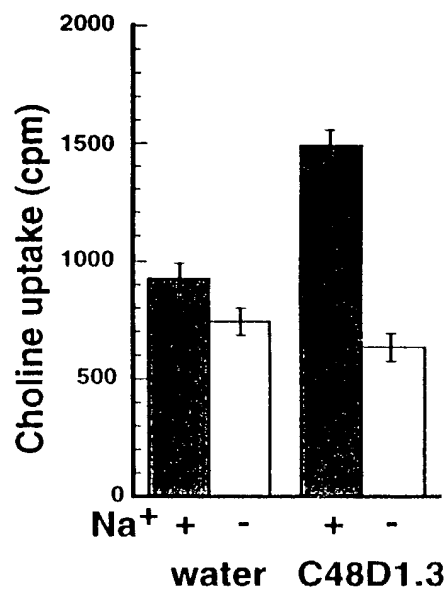


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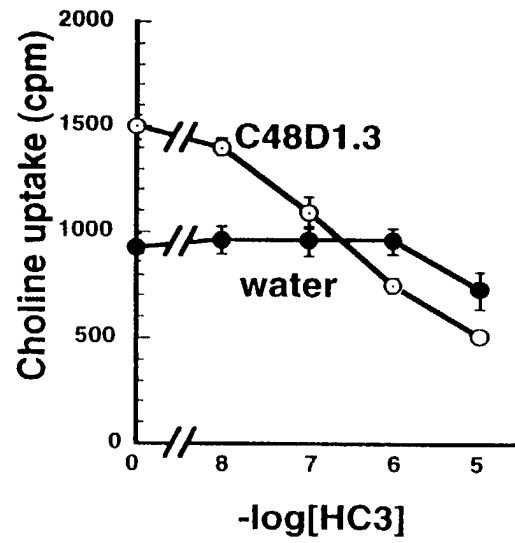


Fig. 4

CHT1	MPFH ¹ EG ² VAI ³ FY ⁴ LI ⁵ VGIWA ⁶ AWK ⁷ IKNS ⁸ ----G ⁹ AEER ¹⁰ SEA ¹¹ VGGR ¹² IGLLVGGF ¹³	56
cht-1	-MAD ¹⁴ LG ¹⁵ VAI ¹⁶ FY ¹⁷ LI ¹⁸ VGIWAGRK ¹⁹ KSSKELES ²⁰ AGAA ²¹ EE ²² LAGR ²³ IGTLVGIF ²⁴	59
I		
CHT1	TMTATWVGGGYINGTAEA ²⁵ YGP ²⁶ GCGLAWAQAP ²⁷ GY ²⁸ SL ²⁹ GGL ³⁰ FAKPMRS ³¹ GY ³² TMLD ³³	116
cht-1	TMTATWVG ³⁴ GAYINGTAEA ³⁵ YNG ³⁶ GLLGCQAP ³⁷ GY ³⁸ SL ³⁹ GGL ⁴⁰ FAKKMRE ⁴¹ GY ⁴² TMLD ⁴³	117
II		
CHT1	PFQ ⁴⁴ IY ⁴⁵ GKR ⁴⁶ GGL ⁴⁷ PALMGEVFWAAAI ⁴⁸ SALGAT ⁴⁹ SVI ⁵⁰ D ⁵¹ DNISVI ⁵² SALIA ⁵³ YT ⁵⁴	176
cht-1	PFQ ⁵⁵ KY ⁵⁶ QR ⁵⁷ GGL ⁵⁸ PALLGETFWTAAI ⁵⁹ SALGAT ⁶⁰ SVI ⁶¹ G ⁶² DNASVT ⁶³ SACIA ⁶⁴ YT ⁶⁵	177
III IV		
CHT1	VGGLY ⁶⁶ SVAYTDVVQLFCIF ⁶⁷ GLW ⁶⁸ SV ⁶⁹ PAF ⁷⁰ SHPV ⁷¹ TDIGFTAVHAKYQSPW ⁷² GTIES ⁷³ -V ⁷⁴	235
cht-1	TGGYY ⁷⁵ SVAYTDVVQLFCIF ⁷⁶ GLW ⁷⁷ CV ⁷⁸ PAA ⁷⁹ VHDGAKDISRNAG ⁸⁰ -----DW ⁸¹ GEIGGFK ⁸²	231
V		
CHT1	EVYTW ⁸³ DN ⁸⁴ LLL ⁸⁵ MGGIPWQAYFQ ⁸⁶ RVLSS ⁸⁷ SATYAQVLSF ⁸⁸ AAFGC ⁸⁹ MA ⁹⁰ PAICIGATG ⁹¹	295
cht-1	ETSLW ⁹² EDC ⁹³ LLL ⁹⁴ MGGIPWQVYFQ ⁹⁵ RVLSS ⁹⁶ KIAHGAQTLSE ⁹⁷ AGVGC ⁹⁸ MA ⁹⁹ PPALIGATA ¹⁰⁰	291
VI VII		
CHT1	ASTDWNQTAYGFPDPKTKEEAD ¹⁰¹ -----M ¹⁰² TP ¹⁰³ EV ¹⁰⁴ QYLCPV ¹⁰⁵ TSF ¹⁰⁶ GLGAVSAAMSSAD ¹⁰⁷	349
cht-1	RNTDWRMTDYSPWNGTKVES ¹⁰⁸ I ¹⁰⁹ PPDKRNM ¹¹⁰ PE ¹¹¹ VFQYLTPR ¹¹² IV ¹¹³ AF ¹¹⁴ GLGAVSAAMSSAD ¹¹⁵	351
VIII		
CHT1	SSL ¹¹⁶ LSA ¹¹⁷ SMFARNIYQLS ¹¹⁸ ERQ ¹¹⁹ AS ¹²⁰ KE ¹²¹ IVVMRI ¹²² IV ¹²³ VFGASATAMALLT ¹²⁴ GVYGLWYLS ¹²⁵	409
cht-1	SSV ¹²⁶ LSA ¹²⁷ SMFARNI ¹²⁸ KL ¹²⁹ IRP ¹³⁰ AS ¹³¹ KE ¹³² IVMRI ¹³³ IV ¹³⁴ CVGIMATIMALT ¹³⁵ IOS ¹³⁶ YGLWYLC ¹³⁷	411
IX		
CHT1	SDLVY ¹³⁸ IT ¹³⁹ FPQLLCV ¹⁴⁰ IP ¹⁴¹ KG ¹⁴² INTYGA ¹⁴³ AGYIFGLFLR ¹⁴⁴ ITGGEPY ¹⁴⁵ YLQP ¹⁴⁶ IFYPGYYPDK ¹⁴⁷	469
cht-1	SDLVY ¹⁴⁸ IT ¹⁴⁹ FPQLLCV ¹⁵⁰ MPR ¹⁵¹ NTYGS ¹⁵² AGYAVGLVRL ¹⁵³ IGGEPL ¹⁵⁴ SLPA ¹⁵⁵ CHYPMYT ¹⁵⁶ ---D ¹⁵⁷	469
X XI		
CHT1	NGIYNQRFPFK ¹⁵⁸ TL ¹⁵⁹ SMY ¹⁶⁰ TSF ¹⁶¹ TNICSYL ¹⁶² AKYLF ¹⁶³ SGTLPP ¹⁶⁴ KL ¹⁶⁵ DI ¹⁶⁶ DAVVS ¹⁶⁷ R---HSEENM ¹⁶⁸	526
cht-1	G ¹⁶⁹ ---VQYF ¹⁷⁰ PFRTTAM ¹⁷¹ SS ¹⁷² MATIIYIVSIQ ¹⁷³ SEKLF ¹⁷⁴ SGRLSP ¹⁷⁵ WDV ¹⁷⁶ MGCVNIPIDH ¹⁷⁷ VPLPS ¹⁷⁸	526
XII		
CHT1	DK ¹⁷⁹ ILVRNENIKL ¹⁸⁰ NELAPVKPRQS ¹⁸¹ TL ¹⁸² SSTF ¹⁸³ INKE ¹⁸⁴ ALLD ¹⁸⁵ VD ¹⁸⁶ SSPEGSGT ¹⁸⁷ EDNLQ ¹⁸⁸	580
cht-1	DV ¹⁸⁹ SPAVSSE ¹⁹⁰ ---TLNMKAPNGTPAP ¹⁹¹ VHPNQPS ¹⁹² DE ¹⁹³ NTLLHPY ¹⁹⁴ SDQSYYS ¹⁹⁵ TNSN ¹⁹⁶ ---	576

Fig. 5

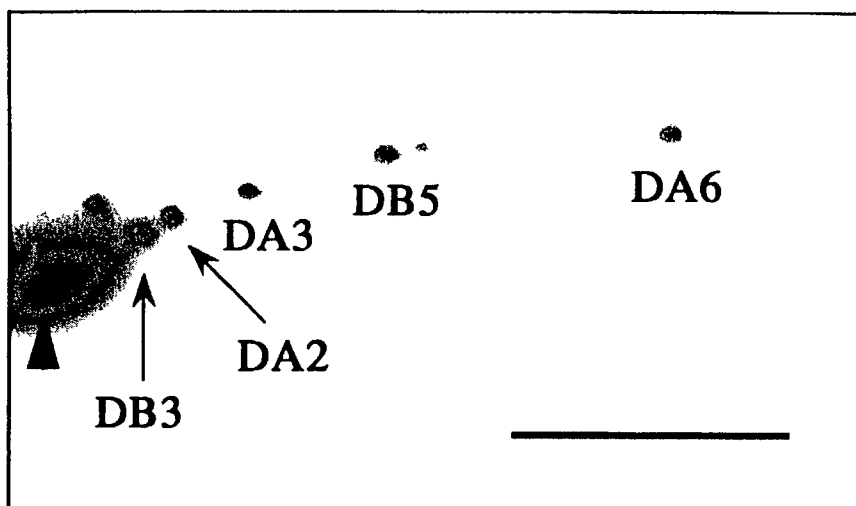


Fig. 6

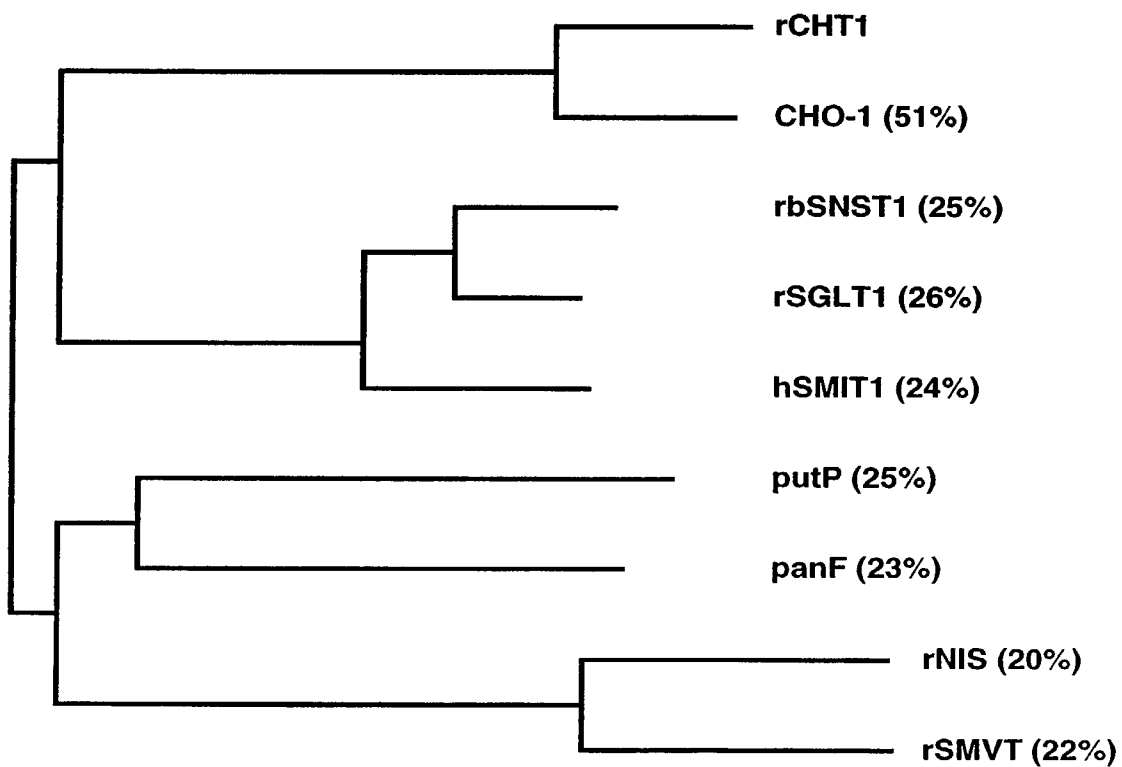


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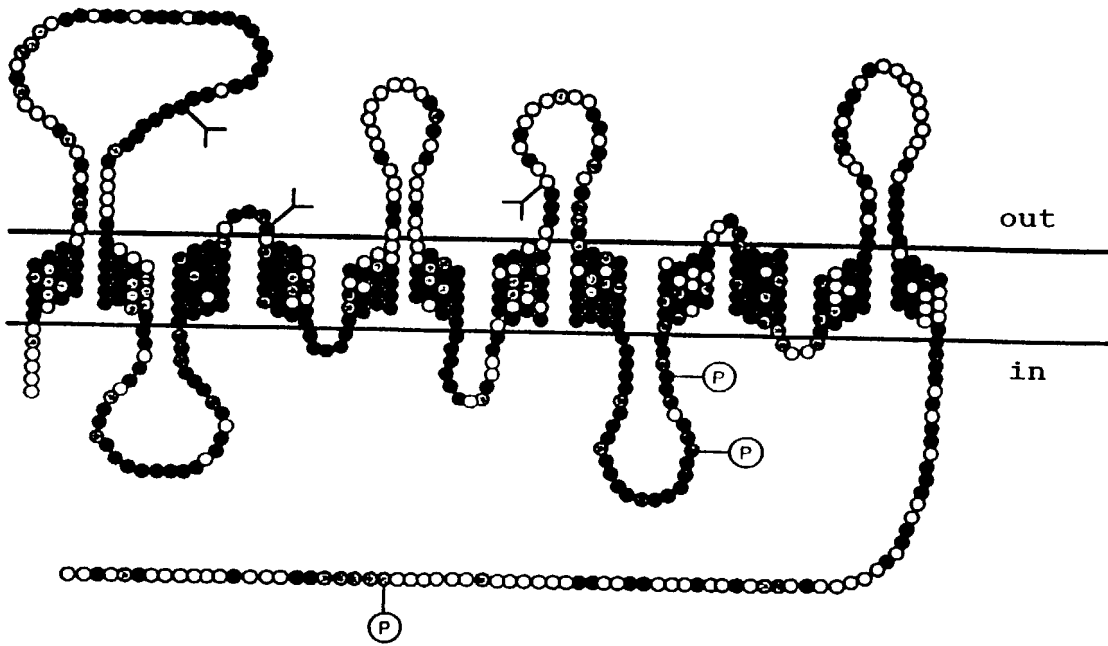


Fig. 8

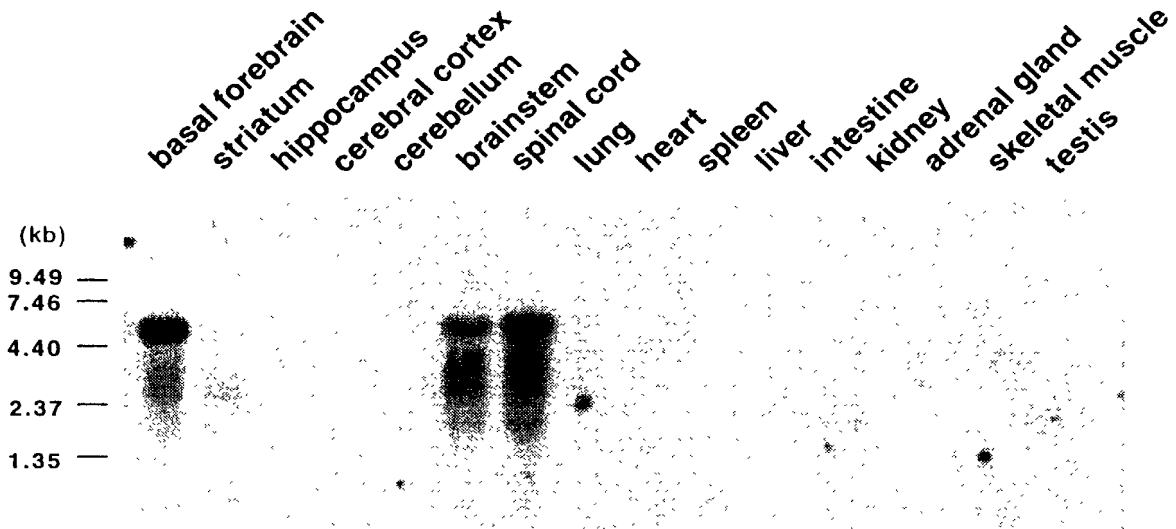


Fig. 9

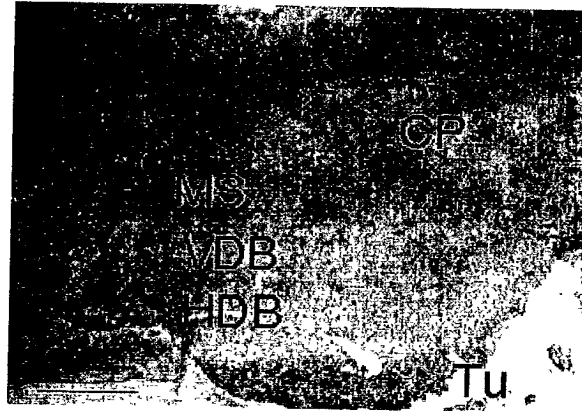


Fig. 10

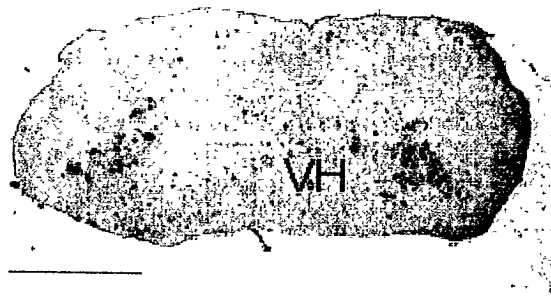


Fig. 11

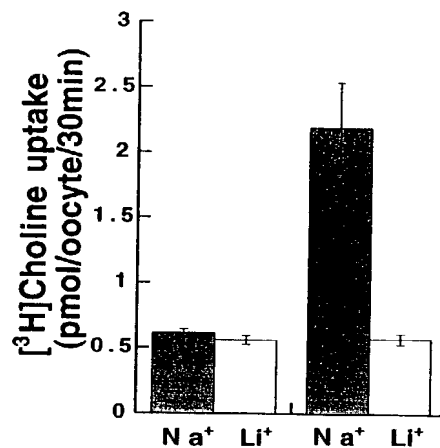


Fig. 12

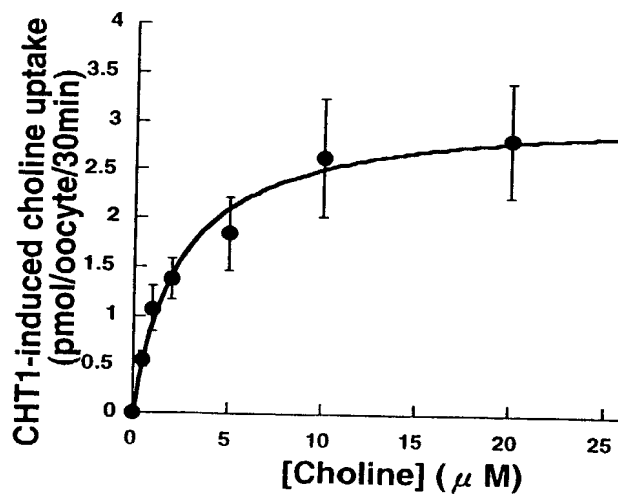


Fig. 13

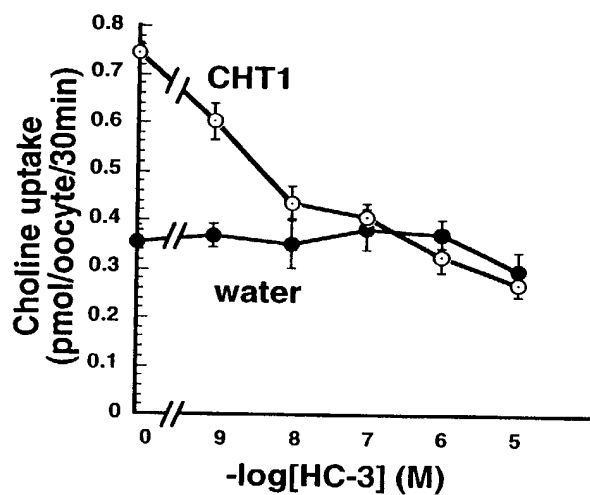


Fig. 14

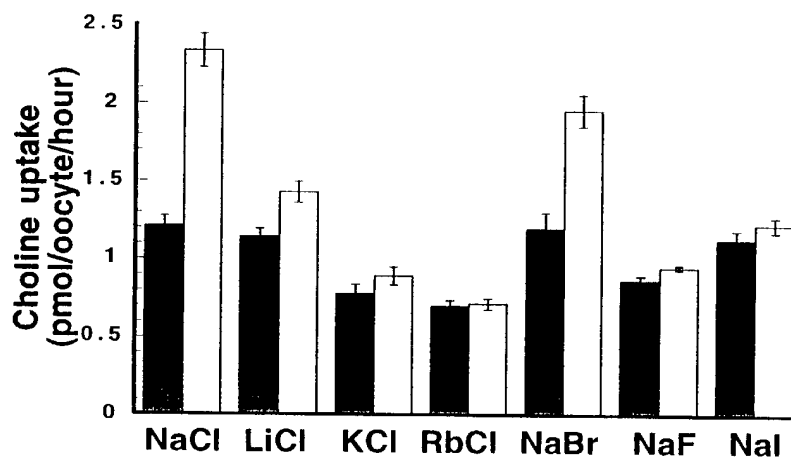


Fig. 15

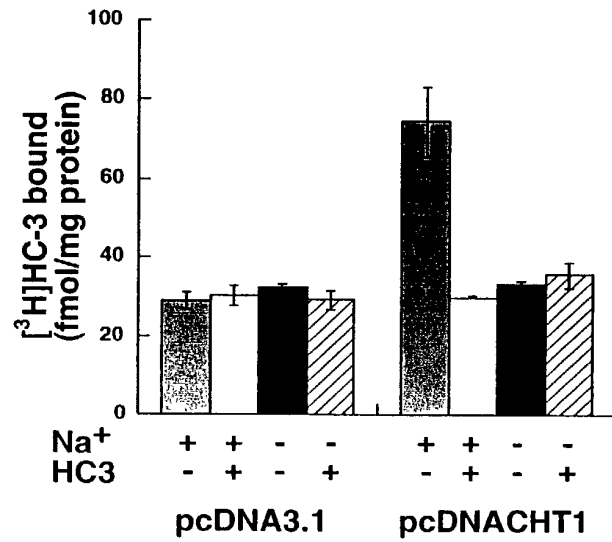


Fig. 16

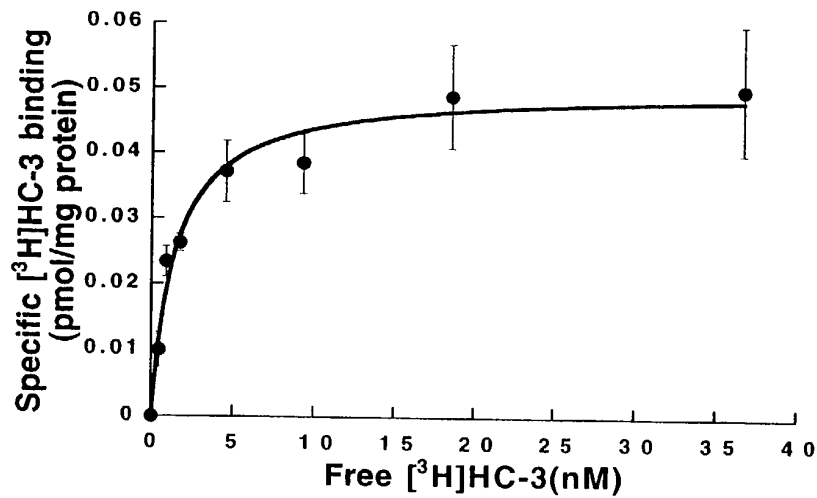
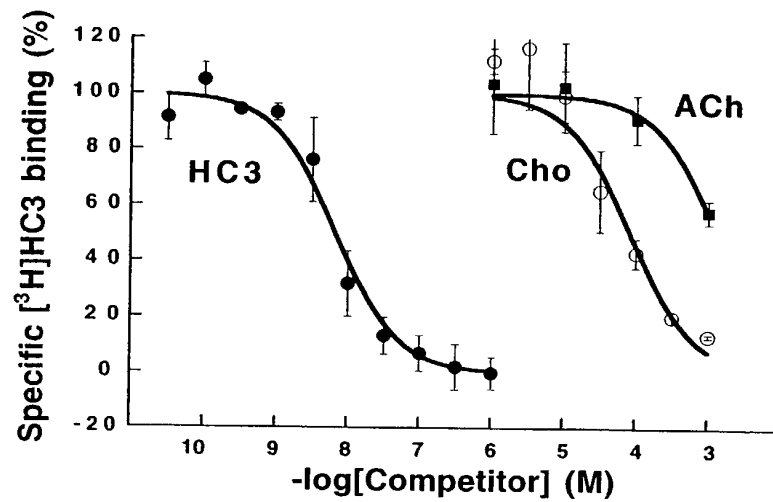


Fig. 17



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<110> JAPAN SCIENCE AND TECHNOLOGY CORPORATION

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Tyr	Gly	Ala	Val	Ala	Gly	Tyr	Ile	Phe	Gly	Leu	Phe	Leu	Arg	Ile	Thr
						435					440				445
Gly	Gly	Glu	Pro	Tyr	Leu	Tyr	Leu	Gln	Pro	Leu	Ile	Phe	Tyr	Pro	Gly
						450					455				460
Tyr	Tyr	Pro	Asp	Lys	Asn	Gly	Ile	Tyr	Asn	Gln	Arg	Phe	Pro	Phe	Lys
465						470					475				480
Thr	Leu	Ser	Met	Val	Thr	Ser	Phe	Phe	Thr	Asn	Ile	Cys	Val	Ser	Tyr
						485					490				495
Leu	Ala	Lys	Tyr	Leu	Phe	Glu	Ser	Gly	Thr	Leu	Pro	Pro	Lys	Leu	Asp
						500					505				510
Ile	Phe	Asp	Ala	Val	Val	Ser	Arg	His	Ser	Glu	Glu	Asn	Met	Asp	Lys

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530	535	540
Val Lys Pro Arg Gln Ser Leu Thr Leu Ser Ser Thr Phe Thr Asn Lys		
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Asp Asn Leu Gln		
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Leu Ile Leu Leu Val Gly Ile Trp Ala Ala Trp Arg Thr Lys Asn Ser

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ggc agc gca gaa gag cgc agc gaa gcc atc ata gtt ggt ggc cga gat 144

Gly Ser Ala Glu Glu Arg Ser Glu Ala Ile Ile Val Gly Gly Arg Asp

35 40 45

att ggt tta ttg gtt ggt gga ttt acc atg aca gct acc tgg gtc gga 192

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360

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Arg Gln Asn Ala Ser Asp Lys Glu Ile Val Trp Val Met Arg Ile Thr

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Val Phe Val Phe Gly Ala Ser Ala Thr Ala Met Ala Leu Leu Thr Lys

385

390

395

400

act gtg tat ggg ctc tgg tac ctc agt tct gac ctt gtt tac atc gtt 1248
Thr Val Tyr Gly Leu Trp Tyr Leu Ser Ser Asp Leu Val Tyr Ile Val

405

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Ile Phe Pro Gln Leu Leu Cys Val Leu Phe Val Lys Gly Thr Asn Thr

420

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Tyr Gly Ala Val Ala Gly Tyr Val Ser Gly Leu Phe Leu Arg Ile Thr

435

440

445

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Gly Gly Glu Pro Tyr Leu Tyr Leu Gln Pro Leu Ile Phe Tyr Pro Gly

450

455

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Tyr Tyr Pro Asp Asp Asn Gly Ile Tyr Asn Gln Lys Phe Pro Phe Lys

465 470 475 480
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 Thr Leu Ala Met Val Thr Ser Phe Leu Thr Asn Ile Cys Ile Ser Tyr
 485 490 495
 cta gcc aag tat cta ttt gaa agt gga acc ttg cca cct aaa tta gat 1536
 Leu Ala Lys Tyr Leu Phe Glu Ser Gly Thr Leu Pro Pro Lys Leu Asp
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 gla ttt gat gct gtt gtt gca aga cac agt gaa gaa aac atg gat aag 1584
 Val Phe Asp Ala Val Val Ala Arg His Ser Glu Glu Asn Met Asp Lys
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<213> Homo sapiens

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          20           25           30
Gly Ser Ala Glu Glu Arg Ser Glu Ala Ile Ile Val Gly Gly Arg Asp
          35           40           45
Ile Gly Leu Leu Val Gly Gly Phe Thr Met Thr Ala Thr Trp Val Gly
 50           55           60
Gly Gly Tyr Ile Asn Gly Thr Ala Glu Ala Val Tyr Val Pro Gly Tyr
 65           70           75           80
Gly Leu Ala Trp Ala Gln Ala Pro Ile Gly Tyr Ser Leu Ser Leu Ile
          85           90           95
Leu Gly Gly Leu Phe Phe Ala Lys Pro Met Arg Ser Lys Gly Tyr Val
          100          105          110
Thr Met Leu Asp Pro Phe Gln Gln Ile Tyr Gly Lys Arg Met Gly Gly
          115          120          125
Leu Leu Phe Ile Pro Ala Leu Met Gly Glu Met Phe Trp Ala Ala Ala
          130          135          140
Ile Phe Ser Ala Leu Gly Ala Thr Ile Ser Val Ile Ile Asp Val Asp
          145          150          155          160
Met His Ile Ser Val Ile Ile Ser Ala Leu Ile Ala Thr Leu Tyr Thr
          165          170          175
Leu Val Gly Gly Leu Tyr Ser Val Ala Tyr Thr Asp Val Val Gln Leu
          180          185          190
Phe Cys Ile Phe Val Gly Leu Trp Ile Ser Val Pro Phe Ala Leu Ser
          195          200          205
His Pro Ala Val Ala Asp Ile Gly Phe Thr Ala Val His Ala Lys Tyr
          210          215          220
Gln Lys Pro Trp Leu Gly Thr Val Asp Ser Ser Glu Val Tyr Ser Trp
          225          230          235          240
Leu Asp Ser Phe Leu Leu Leu Met Leu Gly Gly Ile Pro Trp Gln Ala
          245          250          255
Tyr Phe Gln Arg Val Leu Ser Ser Ser Ser Ala Thr Tyr Ala Gln Val

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Gly Gly Tyr Ile Asn Gly Thr Ala Glu Ala Val Tyr Gly Pro Gly Cys
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ggt cta gct tgg gct cag gca ccc att gga tat tct ctg agt cta att 288
Gly Leu Ala Trp Ala Gln Ala Pro Ile Gly Tyr Ser Leu Ser Leu Ile
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tta ggt ggt ctg ttt ttt gcg aaa cct atg cgt tcc aag gga tat gtg 336
Leu Gly Gly Leu Phe Phe Ala Lys Pro Met Arg Ser Lys Gly Tyr Val
100 105 110

act atg tta gac cca ttt caa cag atc tat gga aag cgc atg ggt ggg 384
Thr Met Leu Asp Pro Phe Gln Gln Ile Tyr Gly Lys Arg Met Gly Gly
115 120 125

ctg ctc ttc atc cct gca ctg atg gga gag atg ttc tgg gct gca gca 432
Leu Leu Phe Ile Pro Ala Leu Met Gly Glu Met Phe Trp Ala Ala Ala
130 135 140

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Ile Phe Ser Ala Leu Gly Ala Thr Ile Ser Val Ile Ile Asp Val Asp
145 150 155 160

gtg aac ata tcg gtc att gtc tct gca ctc att gcc att ctt tat acc 528
Val Asn Ile Ser Val Ile Val Ser Ala Leu Ile Ala Ile Leu Tyr Thr
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Leu Val Gly Gly Leu Tyr Ser Val Ala Tyr Thr Asp Val Val Gln Leu
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Phe Cys Ile Phe Ile Gly Leu Trp Ile Ser Val Pro Phe Ala Leu Ser
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Leu Ser Ala Ser Ser Met Phe Ala Arg Asn Ile Tyr Gln Leu Ser Phe			
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aga caa aat gca tca gac aag gaa att gtg tgg gtc atg agg atc act			1152
Arg Gln Asn Ala Ser Asp Lys Glu Ile Val Trp Val Met Arg Ile Thr			
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gtg ctt gtg ttc gga gca tct gca aca gcc atg gct ttg ctg acg aag			1200
Val Leu Val Phe Gly Ala Ser Ala Thr Ala Met Ala Leu Leu Thr Lys			
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act gtg tat ggg ctc tgg tac ctg agc tct gac ctt gtc tac atc atc			1248
Thr Val Tyr Gly Leu Trp Tyr Leu Ser Ser Asp Leu Val Tyr Ile Ile			
405	410	415	
atc ttc cca cag ctg ctc tgt gta ctc ttc atc aaa gga acc aac act			1296
Ile Phe Pro Gln Leu Leu Cys Val Leu Phe Ile Lys Gly Thr Asn Thr			
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Tyr Gly Ala Val Ala Gly Tyr Ile Phe Gly Leu Phe Leu Arg Ile Thr			
435	440	445	
gga gga gag cca tat cta tac ttg cag ccc tta atc ttc tac cct ggt			1392
Gly Gly Glu Pro Tyr Leu Tyr Leu Gln Pro Leu Ile Phe Tyr Pro Gly			
450	455	460	
tat tac tct gac aag aat ggt ata tac aat cag agg ttc cca ttt aaa			1440
Tyr Tyr Ser Asp Lys Asn Gly Ile Tyr Asn Gln Arg Phe Pro Phe Lys			
465	470	475	480
act ctc tcc atg gtt acc tca ttc ttt acc aac att tgt gtt tct tat			1488

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Thr Leu Ser Met Val Thr Ser Phe Phe Thr Asn Ile Cys Val Ser Tyr
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Leu Ala Lys Tyr Leu Phe Glu Ser Gly Thr Leu Pro Pro Lys Leu Asp
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gta ttt gat gct gtt gtc gca agg cac agt gaa gag aac atg gac aag 1584
Val Phe Asp Ala Val Val Ala Arg His Ser Glu Glu Asn Met Asp Lys
515 520 525

acc att cta gtc aga aat gaa aat atc aaa tta aat gaa ctt gca cct 1632
Thr Ile Leu Val Arg Asn Glu Asn Ile Lys Leu Asn Glu Leu Ala Pro
530 535 540

gtg aaa cct cgg cag agc cta acc ctc agt tca act ttc acc aat aag 1680
Val Lys Pro Arg Gln Ser Leu Thr Leu Ser Ser Thr Phe Thr Asn Lys
545 550 555 560

gag gcc ctc ctt gat gtt gat tcc agt ccg gag ggg tct ggg act gaa 1728
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Asp Asn Leu Gln
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20	25	30	
Gly Asn Pro Glu Glu His Ser Glu Ala Ile Ile Val Gly Gly Arg Asp			
35	40	45	
Ile Gly Leu Leu Val Gly Gly Phe Thr Met Thr Ala Thr Trp Val Gly			
50	55	60	
Gly Gly Tyr Ile Asn Gly Thr Ala Glu Ala Val Tyr Gly Pro Gly Cys			
65	70	75	80
Gly Leu Ala Trp Ala Gln Ala Pro Ile Gly Tyr Ser Leu Ser Leu Ile			
85	90	95	
Leu Gly Gly Leu Phe Phe Ala Lys Pro Met Arg Ser Lys Gly Tyr Val			
100	105	110	
Thr Met Leu Asp Pro Phe Gln Gln Ile Tyr Gly Lys Arg Met Gly Gly			
115	120	125	
Leu Leu Phe Ile Pro Ala Leu Met Gly Glu Met Phe Trp Ala Ala Ala			
130	135	140	
Ile Phe Ser Ala Leu Gly Ala Thr Ile Ser Val Ile Ile Asp Val Asp			
145	150	155	160
Val Asn Ile Ser Val Ile Val Ser Ala Leu Ile Ala Ile Leu Tyr Thr			
165	170	175	
Leu Val Gly Gly Leu Tyr Ser Val Ala Tyr Thr Asp Val Val Gln Leu			
180	185	190	
Phe Cys Ile Phe Ile Gly Leu Trp Ile Ser Val Pro Phe Ala Leu Ser			
195	200	205	
His Pro Ala Val Thr Asp Ile Gly Phe Thr Ala Val His Ala Lys Tyr			
210	215	220	
Gln Ser Pro Trp Leu Gly Thr Ile Glu Ser Val Glu Val Tyr Thr Trp			
225	230	235	240
Leu Asp Asn Phe Leu Leu Leu Met Leu Gly Gly Ile Pro Trp Gln Ala			
245	250	255	
Tyr Phe Gln Arg Val Leu Ser Ser Ser Ser Ala Thr Tyr Ala Gln Val			
260	265	270	
Leu Ser Phe Leu Ala Ala Phe Gly Cys Leu Val Met Ala Leu Pro Ala			
275	280	285	

Ile Cys Ile Gly Ala Ile Gly Ala Ser Thr Asp Trp Asn Gln Thr Ala
 290 295 300
 Tyr Gly Tyr Pro Asp Pro Lys Thr Lys Glu Glu Ala Asp Met Ile Leu
 305 310 315 320
 Pro Ile Val Leu Gln Tyr Leu Cys Pro Val Tyr Ile Ser Phe Phe Gly
 325 330 335
 Leu Gly Ala Val Ser Ala Ala Val Met Ser Ser Ala Asp Ser Ser Ile
 340 345 350
 Leu Ser Ala Ser Ser Met Phe Ala Arg Asn Ile Tyr Gln Leu Ser Phe
 355 360 365
 Arg Gln Asn Ala Ser Asp Lys Glu Ile Val Trp Val Met Arg Ile Thr
 370 375 380
 Val Leu Val Phe Gly Ala Ser Ala Thr Ala Met Ala Leu Leu Thr Lys
 385 390 395 400
 Thr Val Tyr Gly Leu Trp Tyr Leu Ser Ser Asp Leu Val Tyr Ile Ile
 405 410 415
 Ile Phe Pro Gln Leu Leu Cys Val Leu Phe Ile Lys Gly Thr Asn Thr
 420 425 430
 Tyr Gly Ala Val Ala Gly Tyr Ile Phe Gly Leu Phe Leu Arg Ile Thr
 435 440 445
 Gly Gly Glu Pro Tyr Leu Tyr Leu Gln Pro Leu Ile Phe Tyr Pro Gly
 450 455 460
 Tyr Tyr Ser Asp Lys Asn Gly Ile Tyr Asn Gln Arg Phe Pro Phe Lys
 465 470 475 480
 Thr Leu Ser Met Val Thr Ser Phe Phe Thr Asn Ile Cys Val Ser Tyr
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 Val Phe Asp Ala Val Val Ala Arg His Ser Glu Glu Asn Met Asp Lys
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 Thr Ile Leu Val Arg Asn Glu Asn Ile Lys Leu Asn Glu Leu Ala Pro
 530 535 540
 Val Lys Pro Arg Gln Ser Leu Thr Leu Ser Ser Thr Phe Thr Asn Lys
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565
Asp Asn Leu Gln
580

570

575

Fig. 9

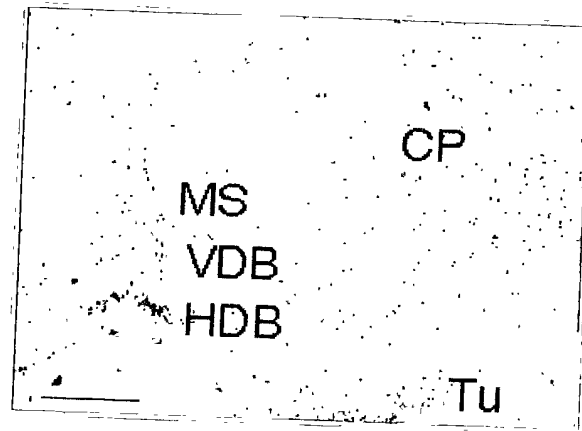


Fig. 10

